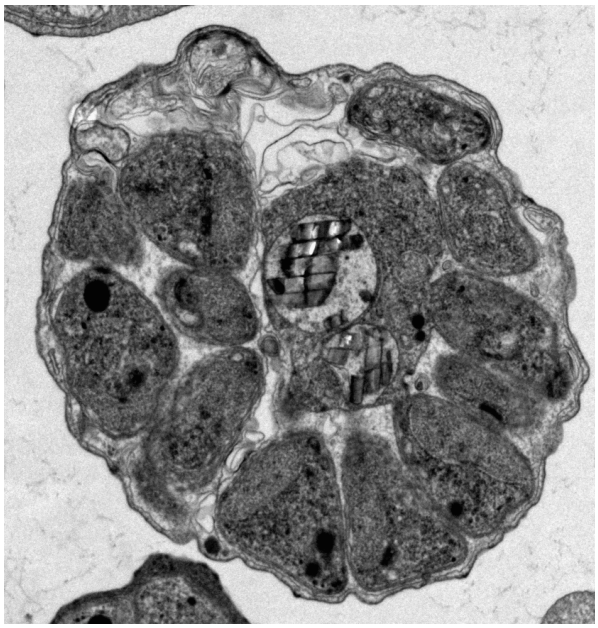
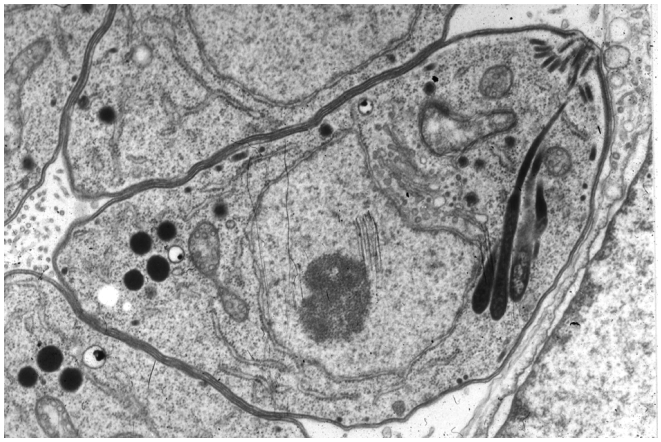
# Middle East Biology of Parasitism 2017

## Module II

## Aspartyl proteases in *Toxoplasma gondii* and *Plasmodium falciparum*





**Dominique Soldati-Favre** (dominique.soldati-favre@unige.ch)
**Sunil Kumar Dogga** (sunil.dogga@unige.ch)
**Karine Frénal** (karine.frenal@unige.ch)
**Paco Pino** (Paco.Pino@unige.ch)

University of Geneva

## Goals of the module

- Build up a working hypothesis based on minimal information
- Design experimental strategies to confirm or refute the hypothesis
- Familiarize with the inducible systems to investigate the function of essential genes and the diverse assays to elucidate gene function

**Exploit the strategies available and the experimental tools to investigate gene function**

The objectives are to learn how to:

- Propagate and manipulate the parasites (theory only)
- Identify by immunofluorescence assay the subcellular compartments of the parasite
- Familiarize with the assays available to perform a phenotypic analysis of parasite mutants

**Experiment 1:** Extract information about *Toxoplasma gondii* Aspartyl protease 3 (TgASP3) and design the strategy to determine the function of TgASP3 in *T. gondii*.

**Experiment 2:** Immunofluorescence assays to quantify intracellular growth, egress, and invasion capabilities of the parasites and to visualize the different organelles.

**Experiment 3:** Identification of potential substrate proteins of the protease being studied and investigation by western blots in *T. gondii* and *P. falciparum*

## Lab Schedule: overview

**Monday July 31st**

Morning:

Introduction to the module
Genome database and literature mining from ToxoDB/PlasmoDB/EuPathDB
Formulation of the working hypothesis
Design of the experimental approaches

Afternoon:

Introduction to parasite culture and assays
Immunofluorescence assays (IFAs) : to check for organelles, and to quantify invasion, egress, and intracellular growth of the parasites

**Tuesday August 1st**

Observation of the IFAs and recording the results
Western blot on protein lysates of the parasite: loading and running the gel, transferring the protein to blotting membrane and blocking the membrane
Discussion

**Wednesday August 2nd**

Observation of the IFAs and recording the results
Western blot: Continuation of the western blot – antibody staining (primary and secondary) and membrane development for specific protein detection
Discussion

**Thursday August 3rd**

Observation of the IFAs and recording the results
Work on presentation

**Friday August 4th**

Presentations and discussions

## MeBoP Module – Detailed Protocols

A. Culturing HFF (Human Foreskin Fibroblast) cells and *T. gondii*
B. Culturing Vero (African Monkey Kidney) cells and *T. gondii*
C. Transfection of *T. gondii*
D. Plaque Assay
E. Immunofluorescence Assay (IFA)
F. Intracellular Growth Assay
G. Ionophore Induced Egress Assay
H. Invasion assay
I. SDS-PAGE gel running, western blotting
J. Parasite strains

# A. Culturing HFF (Human Foreskin Fibroblast) cells

**Reagents:**

- 10% FCS Media (500ml)
  450 ml DMEM (Dulbecco's Modified Eagle Medium) high glucose, no glutamine
  50 ml FCS (fetal calf serum)
  2 mM L- Glutamine
  25 µg/ml Gentamycin

- 5% FCS Media (500ml)
  485 ml DMEM
  25 ml FCS
  2 mM L-Glutamine
  25 µg/ml Gentamycin

- 0.25% Trypsin/1mM EDTA

**Splitting/Passaging HFF cells:**

1. Add 0.25% Trypsin/EDTA to a confluent monolayer of HFF (3 ml for a T175 flask)
2. Incubate for 5 min at 37°C
3. To release the cells from the plate gently tap the bottom of the dish. Check under the microscope to see if all of the cells have detached before continuing
4. Resuspend cells in 10% media

**Culture of *Toxoplasma gondii*:**

When the HFF cells are confluent, replace the 10% media with 5% media. The cells will remain happy for a couple of weeks, but will not continue to grow (due to contact inhibition). They can be used now for infection with *T. gondii* parasites.
Note, the amount of parasites used for infecting a new monolayer of HFF allows to estimate at which point the HFF will be fully lysed by the parasites. For example, if ~300 µl of freshly egressed parasites from a 6cm dish are passed into a new 6 cm dish, it will take 2 days before the HFF of the new dish are fully lysed.

## B. Culturing Vero (African monkey kidney) cells

**Reagents:**

- 5% Media
- 0.25% Trypsin/EDTA

**Splitting Vero cells:**

1. Add 0.25% Trypsin/EDTA to a 80-100% confluent monolayer of Vero cells (1 ml/T25 flask, 2 ml/T75 flask). Incubate for 5 min at 37°C
2. For cell detachment, gently tap the bottom of the dish and check under the microscope to see if all the cells are detached before continuing
3. Transfer the trypsinized cells to a 15 ml falcon tube and add an equal volume of 5% media. Spin 1500 rpm, 10 min
4. Resuspend cells in 5% media.
5. Plate the cells into a new flask or dish

**Culture of *Toxoplasma gondii*:**

When the Vero cells are 50-80% confluent, they can be infected with parasites. Do not let the Vero cells reach complete confluency because they do not stop growing when a monolayer is formed like HFF but make multilayers (no contact inhibition).
Vero cells are only used to perform large-scale experiments and not for routine passage because they are highly susceptible to mycoplasma contamination. In addition, selection (antibiotic or selection marker) of newly transfected parasite is not optimal in these cells as the Vero will continue to grow whereas only a small fraction of parasite will survive the selection process.

## C. Transfection of *Toxoplasma gondii* by electroporation

**Cytomix:**

| **FOR 500ml** | Stock Solution | Volume/Amount to add | Final Concentration |
|---|---|---|---|
| KCl | 1 M | 60 ml | 120 mM |
| $CaCl_2$ | 1 M | 75 ml | 0.15 mM |
| $K_2HPO_4/KH_2PO_4$ pH 7.6 | 100 mM * | 50 ml | 10 mM |
| HEPES pH 7.6 | 1 M | 12.5 ml | 25 mM |
| EDTA | Weigh in | 0.372 g | 2 mM |
| $MgCl_2$ | 1 M | 2.5 ml | 5 mM |

* To make 1 liter of 100 mM stock of $K_2HPO_4/KH_2PO_4$ pH 7.6: mix 86.6 ml of 1 M $K_2HPO_4$ with 13.4 ml of 1 M $KH_2PO_4$, fill up with water to 1 liter

To adjust the pH to 7.6 use KOH, cytomix has to be Na free!!
Filter the cytomix solution under the hood and keep it at $4^0$C

**Protocol:**

A. Prepare the parasites to be transfected:
Infect a confluent 6 cm dish with 0.5 ml of freshly egressed tachyzoites 2 days prior the experiment and wash a few hours later. One completely lysed 6 cm dish produces enough parasites for 4 transfections.

B. The day of the transfection, prepare a HFF dish with fresh medium to receive the transfected parasites and get the plasmid DNA to be transfected.
For transient transfection: 20-30 µg of circular plasmid DNA
For stable transfection of a second copy: 60-80 µg of linearized (in the backbone) plasmid
For single or double homologous integration: 15-30 µg of linearized plasmid DNA.

D. Transfection protocol:
1. Harvest the freshly lysed parasites and centrifuge them in a 15 ml falcon tube at 1000g, 10 min in a swing bucket rotor.
2. Resuspend the parasite pellet in 2.5 ml of Cytomix supplemented with 30 µl ATP [2mM] and 30 µl GSH [5mM] per ml of Cytomix.
Transfer 700 µl of resuspended parasites into the 4 mm electroporation cuvette and add the DNA.
3. Electroporation using the settings *T. gondii* in the BTX ElectroCell Manipulator.
(ex: for the BTX ECM 630: voltage 2000V, resistor 50Ω, capacitor 25 µF)
4. Immediately after electroporation add the transfected parasites to the new dish
5. Start the selection process the day after.

## D. Plaque Assays

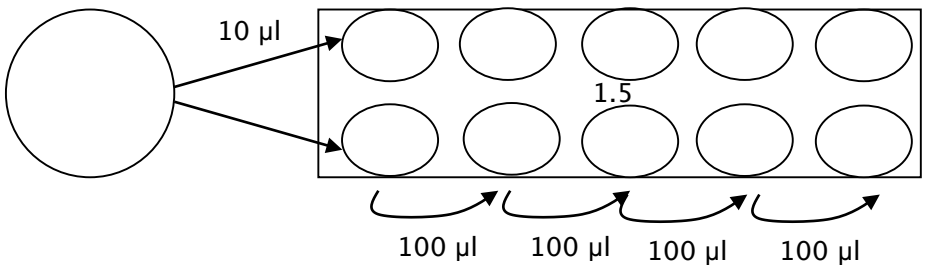
**Reagents:**

- Crystal violet
- 4% Paraformaldehyde (PFA)

This assay will reveal if the parasites are able or not to be propagated in culture. It will give you a general idea of the fitness but you will not know which step of the lytic cycle is affected. Here only the size of the plaques is informative, not their number. Set up duplicate dishes or wells for each condition.

**Protocol:**

1. Prepare the HFF plate to receive the parasites: add to each well fresh medium and ± drug (if necessary).
2. Transfer 10 µl of parasites from a totally lysed dish into the first well of an IFA plate and pass 100 µl to the neighboring well. Continue the dilution process up to the end of the line.




3. Incubate for 7-8 days without disturbing the wells (don't bump them, move them or slam incubator doors).
4. After incubation for 7-8 days, aspirate the medium, rinse the infected monolayer with PBS, fix for 10 minute with 4% paraformaldehyde, and stain with crystal violet for 15 min (1/5 dilution of crystal violet in water, filter the solution with 0.22 µm filters).
5. Aspirate the crystal violet solution, rinse twice with water and let it dry.

## E. Immunofluorescence Assay (IFA)

**Reagents:**

- 4% paraformaldehyde (PFA) or PFA + 0.005 % Glutaraldehyde (PFA/GA) in PBS, depending on the antibodies used
- 1X PBS/0.1M glycine
- 1X PBS/0.2% (w/v) Triton X-100
- 2%BSA/1X PBS/0.2% (w/v) Triton X-100
- Primary antibodies
- Secondary antibodies
- DAPI
- Fluoromount G

**Protocol:**

1. Remove the medium from an infected HFF-IFA (24well plate with HFF monolayers on coverslips)and fix with PFA or PFA/GA for 10 min (250µl of fixative per well is sufficient).
2. Quench the reaction by adding 1X PBS/0.1M glycine, incubate for 5 min
3. Permeabilize with 1X PBS/0.2% Triton X-100, 20 min on the shaker
4. Block with 2%BSA/1X PBS/0.2% Triton X-100, 20 min on shaker
5. Incubate with primary antibodies in 2%BSA/1X PBS/0.2%Triton (dilution depends on the antibody used) for 1 h (250 µl/well)
6. Wash 3 times with 1X PBS (5-10 min each wash)
7. Incubate with secondary antibodies (1:3000 dilution; 250µl/well), 45 min.
   The plate should be kept in the dark from now on!
8. Wash 3 times with 1X PBS (5-10 min each wash)
9. Stain cells with DAPI for 5-10 min.
10. Wash with 1X PBS
11. Carefully mount coverslips on the slide with cell layer facing down using a drop of mounting solution (Fluoromount G)

Keep slides in the dark at RT in order to let them dry. Then check them using a fluorescence microscope. For long-term storage, keep them at 4°C, in the dark.

## F. Intracellular growth/replication assay

*T. gondii* divides synchronously within a vacuole but asynchronously across the different vacuoles. Counting the number of parasites/vacuole across several vacuoles is thus an easy and efficient way to determine the replication rate of the parasites.

**Reagents:**

- ATc
- PFA/GA
- α-GAP45
- Secondary anti-rabbit
- PBS
- Triton-X100
- BSA

**Protocol:**

1. Transfer 10-15 µl of parasites from a fully lysed dish to wells of an IFA plate
2. Incubate for 24-30 hr, 37°C
3. Fix with PFA/GA.
4. Perform IFA using α-GAP45 Abs and secondary α-rabbit Abs and count the number of parasites/vacuole (only vacuoles with at least 2 parasites are counted). Count 100 vacuoles per coverslip.

The experiment should be done at least 3 times to have standard deviations.

## G. Ionophore Induced Egress Assay

In *T. gondii*, egress is an active process relying on the parasites' ability to sense that their host cell is dying. Treatment with the $Ca^{2+}$ ionophore (such as A23187) leads to an increase in the intra-parasitic level of $Ca^{2+}$, which artificially induces parasite egress as early as two hours post-infection. During this process, the conoid protrudes, the micronemes discharge their content and the parasites become motile.

**Reagents:**

- Calcium ionophore A23187 (from *Streptomyces chartreusensis*)
- DMEM w/o serum
- DMSO
- ATc
- PFA/GA
- α-GAP45
- α-GRA3
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and Triton-X100
- BSA

**Protocol:**

1. Resuspend parasites from a 6 cm dish.
2. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium).
3. Inoculate IFA plate with 30-50 µl of the diluted parasites and incubate for 30 h at 37°C. Don't forget to plate for a control well.
4. Aspirate the medium and wash with DMEM **w/o serum**
5. Incubate the cells with DMEM **w/o serum** containing either 3 µM A23187 or DMSO, for 8-10 min, 37°C
6. Fix the cells with PFA/GA and perform IFA with α-GAP45 that stain the parasites and α-GRA3 Abs that stain the parasitophorous vacuole.

## H. Invasion assay

In *T. gondii*, invasion, like egress, is an active process. This process follows sequential steps: (i) microneme discharge and attachment of the parasite to the host cell, (ii) apical reorientation of the parasite, (iii) rhoptries secretion within the host cell, iv) formation of the moving junction and (v) translocation of the junction propelling the parasite forward inside the host cell.
The principle of the assay consists of letting the parasite invade for 30 min before fixation with PFA. Then, antibody incubation is performed without permeabilization, staining the parasite with a surface marker. The absence of permeabilization will block the antibodies outside the host cell, leaving the intracellular parasites unlabelled. Samples will then be permeabilized and stained with a second marker to label the total population. The invasion rate can be then be easily calculated as a ratio of the parasites inside (single stained) to the total (single stained + double stained parasites).

**Reagents:**

- ATc
- PFA/GA
- 1% formaldehyde in PBS
- α-GAP45
- α-SAG1
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and Triton-X100 (TX100)
- BSA

**Protocol:**

A. Resuspend parasites from a 6 cm dish.
B. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium)
C. Inoculate IFA plate with 100 µl of the diluted parasites and incubate for 30 min (± drug) at 37°C.
D. Fix the cells with PFA/GA. **Do not wash the cell** otherwise you can detach non-invaded parasites.
E. IFAs are then process as follow:
    1. without triton!!
        - blocking for 30 min with 2% BSA/PBS
        - incubation for 20 min with α-SAG1 diluted in 2% BSA/PBS
        - 3 washes of 5 min in PBS.
    2. Cells were then fixed with 1% formaldehyde for 7 min and washed once with PBS.
    3. without triton!!
        - permeabilization for 20 min using 0.2% TX100/PBS
        - incubation for 30 min with α-GAP45 diluted in 2% BSA/0.2% TX100/PBS
        - 3 washes with 0.2% Triton X-100/PBS
        - incubation with secondary antibodies for 30 min.
        - 3 washes with 0.2% Triton X-100/PBS
        - DAPI staining and mounting

# I. SDS-PAGE gel running, western blotting

**Preparation of parasite protein lysate:**

A fully lysed well from a 6 well plate produces enough parasites ($2x10^7$) to run several gels

1. Harvest the freshly lysed parasites and centrifuge at 1000g, 10 min
2. Wash the parasite pellet in 1X PBS
3. Resuspend the parasite pellet in 100µl 1X PBS
4. Add 80 µl 2xSDS loading buffer and 20 µl of DTT (1 M), boil at 95°C for 10 min
5. The lysate can be used directly or stored at -20°C

**SDS-PAGE gels:**

1. Use the precast gels
2. Run each gel at constant 35 mA per gel (approx. 45 minutes running)

**Western Blotting with ECL detection kit**

Transfer the proteins from the gel onto a nitrocellulose membrane with a semi-dry transfer machine

**Reagents:**

- 1xPBS/0.05% Tween20
- Milk powder
- 5% non-fat milk powder in 1X PBS/0.05% Tween20
- Primary antibodies
- Secondary antibodies, HRP conjugated
- ECL plus kit

**Protocol:**

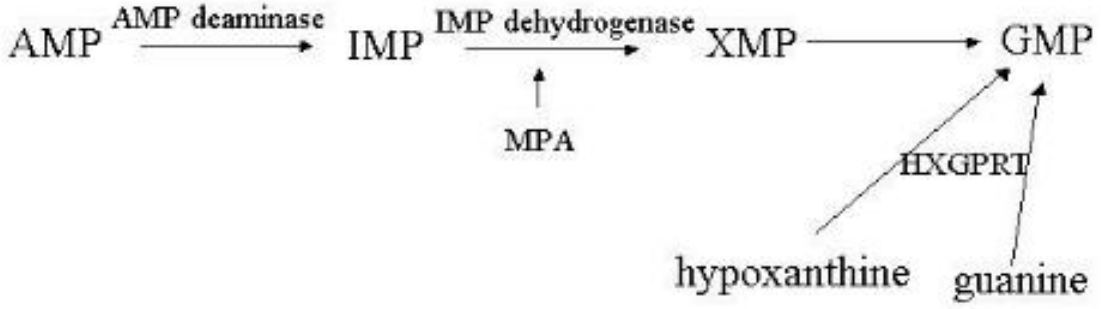
1. Blot the membrane with 5% milk/PBS/Tween solution 45 min, at room temperature (RT) or overnight at 4°C
2. Incubate the membrane with the appropriate dilution of the primary antibody in 5% milk/PBS/Tween for 1 h, RT or overnight at 4°C
3. Wash the membrane 3 times in 1xPBS/0.05% Tween, 10 min each, RT
4. Incubate the membrane with secondary antibodies (1:3000 dilution) in 5% milk/PBS/Tween for 1 h, RT
5. Wash the membrane 4 times in 1xPBS/0.05% Tween, 10 min each, RT
6. Briefly dry the membrane, then incubate it with the ECL plus detection kit
7. Expose to capture the chemiluminescent signal

   Note: For reprobing, the membrane can be stripped by incubating for 1 h, RT with stripping solution (PBS/2%SDS and 7µl/ml of β-Mercaptoethanol). Wash extensively with PBS and PBS/0.05% Tween and re-block membrane with 5% milk before incubation with antibodies.

## J. Parasite strains

### RHΔHX strain

The *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) locus has been deleted from the RH strain and thus can now be used for both positive and negative selection. HXGPRT is not an essential enzyme in *Toxoplasma,* because AMP (adenosine monophosphate) and IMP (inosine monophosphate) are interconvertible, synthesizing guanine nucleotides via AMP deaminase. In the absence of HXGPRT, the parasite depends on the interconversion of AMP into GMP (guanine monophosphate) and the rate-limiting enzyme in this pathway is IMPDH (inosine monophosphate dehydrogenase) which can be inhibited by mycophenolic acid (MPA) treatment. This inhibiton combined with xanthine (XAN) supplementation can thus be used to positively select parasites transfected with a plasmid containing the HXGPRT selection marker, because the parasites will have to rely on the HXGPRT pathway. Alternatively, parasites can be negatively selected with xanthine analogues such as 6-thioxanthine (TX), which converts HXGPRT into a toxic compound. Alternatively, parasites can be selected with chloramphenicol, pyrimethamine or bleomycin.




### RHΔKu80 strain

The most successful method to generate genetic knockouts and incorporate reporter proteins or tags to endogenous loci is via the use of a parasite strain (RHΔHX) in which the *Ku80* gene was disrupted. Ku80 is normally implicated in the non-homologous end-joining repair (NHEJ) machinery of DNA double-strand break. In the *T. gondii ΔKu80* strain, the high rate of random integration is abolished, thus leading almost exclusively to homologue recombination.

## TgASP3-iKD (TATi)

Parasite strain in which *TgASP3* is expressed under the tetracycline-inducible promoter and its expression can therefore be turned off upon addition of anhydrotetracycline (ATc).

**Generation of the tet-inducible ASP3 strains**